University of Alberta

Cloning, expressing and *in vivo* characterization of human ADAR3

by

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ABSTRACT

RNA transcripts undergo multiple processing events to become mature mRNA. These processes include 5′ capping, 3′ processing and polyadenylation, splicing and editing. RNA editing is a chemical modification occurring on nucleotides in RNA, which can change RNA secondary structure, add, delete or alter codons and create splicing sites. Deamination is one way in which nucleotides are modified and there are two types of deamination. The A-to-I deamination is the most prevalent one and has been intensively studied in the last twenty years. The enzymes catalyzing A to I conversion are known as Adenosine Deaminase that act on RNA (ADAR). There are three ADAR proteins existing in humans, ADAR1, ADAR2 and ADAR3. Among the three members, ADAR1 and ADAR2 have been well characterized but ADAR3, the most recently found one, was shown to be catalytically inactive on known substrates of ADAR1 and ADAR2 although the three ADARs have similar domain features. In this thesis, experiments were conducted to clone and over-express ADAR3. Fluorescent microscopy and FRET experiments were conducted to characterize ADAR3 *in vivo*. Editing experiments were also performed to study the activity of ADAR3 deaminase domain.

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CHAPTER 1

Introduction

RNA transcripts undergo multiple processing events to become mature mRNA. These processes include 5′ capping, 3′ processing and polyadenylation, splicing and editing. RNA editing was first discovered in trypanosomes (1) and gradually found in more animals including mammals.

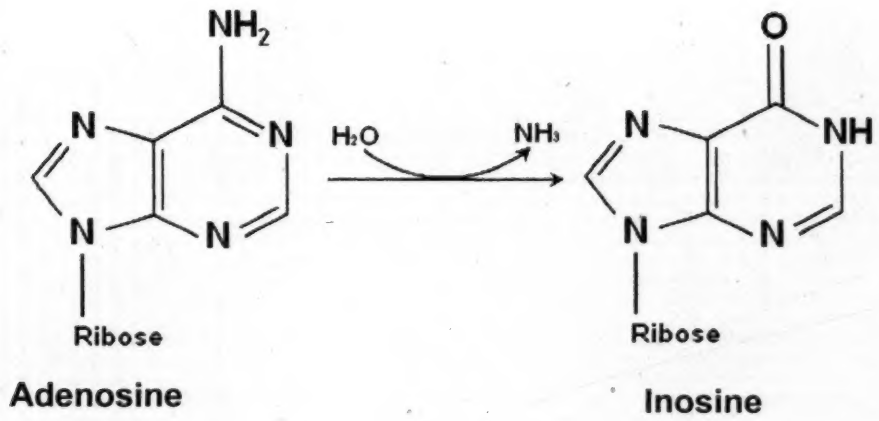
RNA editing is a chemical modification occurring on nucleotides in RNA, which can create, delete or alter the meanings of codons, change RNA secondary structure, or create splicing sites. Deamination is one way in which nucleotides are modified and there are two types of deamination. One is Cytidine (C) deaminated to Uridine (U) and the other is the conversion from Adenosine (A) to Inosine (I), which base pairs with cytidine and is recognized as guanosine by the ribosome or other processing enzymes (Figure 1-1). A to I conversion is the most prevalent one and has been intensively studied in the last twenty years.

1. ADAR ENZYME FAMILY

The enzymes catalyzing A to I conversion are known as Adenosine Deaminase that act on RNA (ADAR). ADAR activity was first discovered in *Xenopus laevis* (2-4) and since then different ADAR proteins have been cloned and characterized in both invertebrates (*Caenorbabditis elegans* and *Drosophila melanogaster*) and vertebrates (*Homo sapiens*).

The number of ADAR genes varies in different species. For example, *Drosophila* has only one ADAR gene (5) and *C. elegans* has two ADAR genes (6). In humans, three ADAR genes have been identified, ADAR1 (7), ADAR2 (8-10) and ADAR3 (11).

A.



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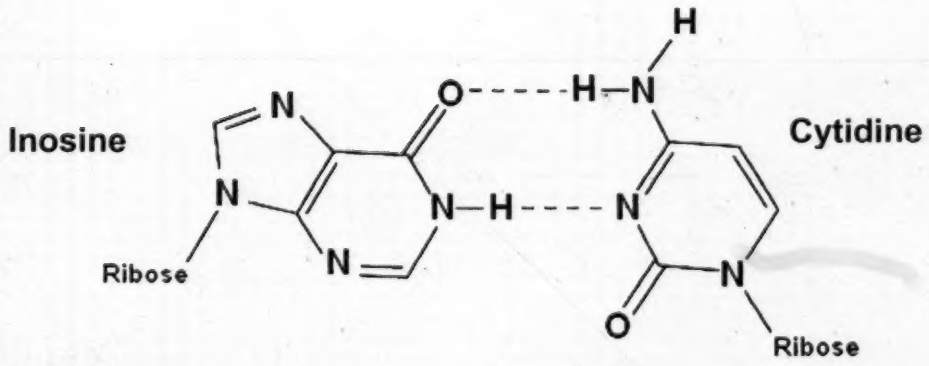

Figure 1-1. Adenosine is converted to inosine by editing. (A) Adenosine is converted to inosine by deamination. (B) Inosine base-pairs with cytidine, which makes inosine recognized as guanosine by ribosome and other processing enzymes.

1.1 Expression and regulation

Human ADAR1 and ADAR2 proteins are expressed in all tissues while the expression of ADAR3 is only detected in the brain and is enriched in certain regions of the brain such as the amygdala and thalamus (11). It has been shown that the expression of ADAR proteins is highly regulated. Two isoforms of human ADAR1

have been identified. A long isoform, ADAR1L, is transcribed from an interferon -inducible promoter on *ADAR1* gene and has been found to be a cytoplasmic/nuclear protein that may be involved in response to viral infection (12, 13). A constitutive short nuclear isoform, ADAR1S, is transcribed from a downstream promoter and by alternative splicing lacks part of the N-terminal region of ADAR1L. ADAR2 expression is regulated by the transcriptional activator, cyclic-AMP-response-element binding (CREB) protein (14). However, the regulation of ADAR3 expression has not been determined.

1.2 Domains and structure

All ADAR proteins share some common domain features, one to three double-stranded RNA binding domains (dsRBD) at the N-terminus and one conserved catalytic domain, the deaminase domain, at the C-terminus (Figure 1-2). As to human ADARs, ADAR1L has two Z-DNA binding domains, Zα and Zβ, at the N-terminus, three dsRBDs and one C-terminal deaminase domain, while ADAR1S has the same pattern of domains but only one Z-DNA binding domain. ADAR2 has two dsRBDs and one deaminase domain and ADAR3 has the same domains as ADAR2 with an extra arginine-rich domain (R domain) at the N-terminus.

The double-stranded RNA binding domain is a 65-70 amino acid structural motif that exists in many proteins that interact with double-stranded RNA and the family is found from prokaryotes to humans and includes diverse proteins: Dicer, Staufen, PKR and ADAR. A crystal structure of dsRBD of *Xenopus laevis* Xlrbpa with dsRNA at 1.9 Å resolution (15) was previously used to model the interaction between ADAR

proteins and double-stranded RNA. The Xlrbpa dsRBD is characterized by an α-β-β-β-α motif, in which two α helices are packed along a face of a three-stranded antiparallel β sheet, and interacts with the minor groove and across the intervening major groove on one face of the dsRNA helix with a total length of 16 bp. The interactions between dsRBD and dsRNA consist of three regions: 1. interaction of the N-terminal α-helix with the RNA minor groove; 2. interaction of the loop between β-strands 1 and 2 with the RNA minor groove; 3. the interaction of the C-terminal α-helix across the RNA major groove. This data suggests that the binding of dsRBD

Figure 1-2. Domain structure of ADAR family. Green box: Z-DNA binding domain; Dark blue box: double-stranded RNA binding domain (dsRBD); Red box: arginine-rich domain; Light blue box: deaminase domain; Yellow region: linker. Human ADARs (hADAR1L- hADAR3), *Drosophila melanogaster* (*Dm*) ADAR, *Caenorbabditis elegans* (*Ce*) ADAR1 and 2 have a similar domain structure.

to double-stranded RNA is dependent on structural features but not sequence. The NMR structure of two dsRBDs of rat ADAR2 was reported in 2006 (16). The two dsRBDs, dsRBD1 and dsRBD2, adopt the α-β-β-β-α motif but differ in the orientation

of the N-terminal α helix and the conformation of the β1-β2 loop. The differences result in the different substrate binding specificity of two dsRBDs. R/G stem-loop RNA was used to map the binding sites of two dsRBDs. dsRBD1 binds near a pentaloop of the substrate and dsRBD2 only binds to the stem with two A-C mismatches in the neighborhood of the editing site interacting with two cytosines (Figure 1-3), which may suggest sequence recognition in the binding of dsRBD to double-stranded RNA. The model shows that two dsRBDs bind to different structural features on R/G stem-loop RNA independently, which suggests the idea that dsRBDs of ADAR proteins play an important role in the recognition and specificity of substrates. The specificity of ADAR proteins will be discussed more below.

ADARs have a conserved C-terminal catalytic domain, the deaminase domain, which catalyzes the deamination reaction on adenosine. The X-ray structure at 1.7 Å resolution of the deaminase domain of human ADAR2 has been reported (Figure 1-4A) (17). The catalytic domain adopts a roughly spherical structure ~ 40 Å in diameter. The active site is indicated by a zinc ion coordinated by H394, C451, C516 and a water molecule bound to E396 by hydrogen bonding (Figure 1-4B). The bound water molecule is thought to displace ammonia during the deamination reaction. The location of active site is in a deep pocket and surrounded by positive electrostatic potential, which probably serves as a binding site of RNA substrates. This deep location of the active site is consistent with the idea that ADARs may use a base-flipping mechanism to expose the C6 of the adenosine for reaction. Interestingly, an IP_6 molecule is found to be buried deep in an extremely basic cavity formed by many arginine and lysine residues at one side of the structure. IP_6 is an abundant

inositol polyphosphate involved in multiple cellular functions including RNA export, DNA repair, chromatin remodeling and endocytosis (18-22) and it is reported to affect AMPA receptors, the coding mRNAs of which are substrates of ADAR2 (23). It has been shown that IP_6 is required for ADAR2 activity and is buried in the cavity during the folding of the domain. Thus, IP_6 may play an important role in the folding process of the catalytic domain.

Besides the dsRBD and the catalytic domain that all ADAR proteins possess, some ADARs have extra domains at the N-terminus which confer the ability to bind to substrates other than double-stranded RNA. The long form of human ADAR1 has two Z-DNA binding domains while the short form only has one. The first Z-DNA binding domain, Zα, is shown to bind to left-handed Z-form double-stranded RNA as well as Z-form DNA (24). Z-form RNA may be the result of viral replication and the binding of Zα domain to Z-RNA may direct ADAR1L to the viral genome, which is consistent with the fact the ADAR1L is inducible by interferon. ADAR3 has an arginine-rich domain, R domain, at its N-terminus. This very basic domain has been suggested to bind to single-stranded RNA although neither double-stranded RNA nor single-stranded RNA substrate of ADAR3 has been demonstrated (11).

2. PHYSIOLOGICAL SIGNIFICANCE OF ADARS

The editing process in the coding regions of mRNAs can alter the amino acid sequences from the one coded by DNA (Table 1) as ADAR converts adenosine to inosine which is recognized by the translational machine and other processing enzymes as guanosine. Besides this, the editing process alters an A:U base pair to I:U

mismatch which may change the structure of double-stranded RNA to affect other processes based on the duplex RNA structure. The ability to alter the coding message of the genome and affect pathways based on RNA structure makes ADARs very important both in invertebrates and vertebrates.

Knock-out experiments on *ADAR* genes have been conducted in various animals from invertebrates to vertebrates. In invertebrates, the knock-out experiments suggest that the ADAR gene is important but not essential, that is, the deletion of ADAR gene may cause defects but is not lethal. For example, *C. elegans* that contain homozygous deletions of both *adr-1* and *adr-2* genes display defective chemotaxis (6); *Drosophila melanogaster* containing a homozygous deletion of the *adar* gene have defects in grooming, mating, tremors and locomotion, which become more severe with age, which suggests defective neural function (5). The situation is very different in mammals in which ADAR genes are essential and the deletions of different ADAR genes are lethal. Mice containing only a single allele of ADAR1 die at embryonic day 14.5 (25) and the homozygous deletion of ADAR2 causes death shortly after birth, between postnatal day 0 to 20 (26).

3. ADAR SUBSTRATES

The possession of dsRBDs confers on ADAR proteins the ability binding double-stranded RNA as substrates. There are many intrinsically formed dsRNAs that are potential substrates of ADARs. One type of dsRNA is formed in pre-mRNAs between an exon and an exon complementary sequence (ECS) in an adjacent or distant intron. The dsRNA formed by the genome or antigenome of a RNA virus can

be one of the targets of ADARs too. The intrinsic hairpin RNA of precurser microRNA is also a potential ADAR target.

3.1 Editing in the coding regions

Glutamate receptor mRNAs

There are multiple glutamate receptor (GluR) subunits and four subunits assemble together to form glutamate-gated ion channels, which conducts fast excitatory neurotransmission in the brain. Multiple editing sites on mRNAs of glutamate receptor subunits which occur in the coding regions have been identified (27, 28). Among all the editing sites, the Q/R site on GluR-B, which changes a glutamine (Q) to arginine (R) located in the loop forming the channel pore (Figure 1-5), is considered the most important: 99% of GluR-B mRNA is the edited form. The change lowers the Ca^{2+} permeability of the channel. Mice with a reduced level (29) or completely lacking editing at this site (26) are born with epilepsy and die about three weeks after birth.

Serotonin receptor mRNAs

Serotonin is a neurotransmitter that binds to a large family of receptors. The mRNA of one subtype of serotonin receptor, 5-HT_{2C}R, which is a member of the G protein-coupled receptor superfamily is the target of ADAR. As shown in Table 1, five editing sites have been identified in the coding region of the mRNA (30, 31). The altering of amino acids caused by editing of the mRNA of this receptor results in the

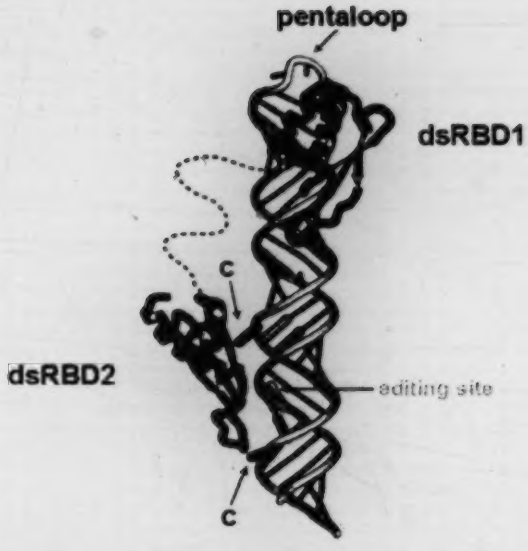

Figure 1-3. dsRBD1 and 2 of human ADAR2 bind to different regions of R/G stem-loop RNA. dsRBD1 binds to the pentaloop and dsRBD2 specifically binds to the stem with two A-C mismatches in the neighborhood of the editing site. (Figure is adapted from Stefl R et al., Structure 2006, 14: 345-355)

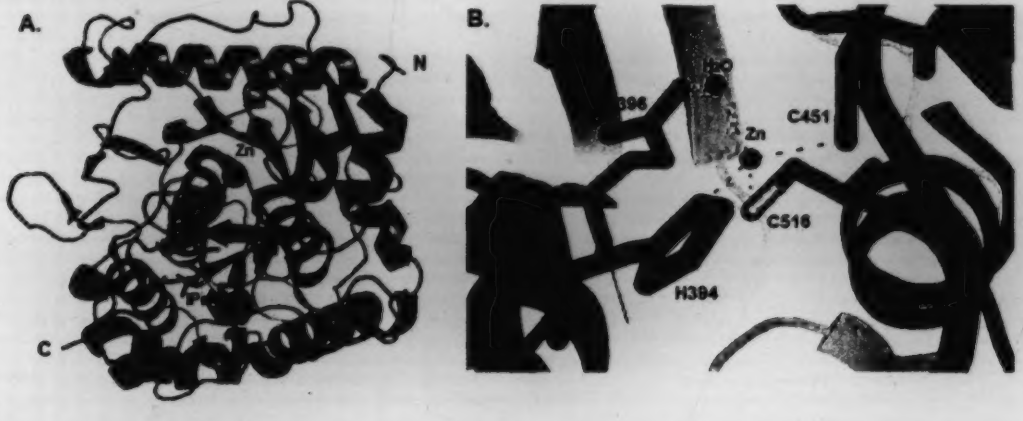

Figure 1-4. The X-ray structure of the catalytic domain of human ADAR2. (A) The ribbon model of the catalytic domain of human ADAR2. (B) Residue interactions at the active site. Zinc ion, coordinating residues (H394, E396, C451, C516) and the nucleophilic water are shown.

	RNA	Codon changes
Human	GluR-B	Q/R, R/G
	GluR-C	R/G
	GluR-D	R/G
	GluR-5	Q/R
	GluR-6	Q/R, I/V, Y/C
	Serotonin receptor	I/V, I/M, N/D, N/S, N/G
HDV	HDV antigenome	Amber/W
Drosophila	Calcium channel	S/G, M/I, N/S, M/V, N/D, N/G, R/G
	Sodium channel	Q/R, Y/C, M/V, N/D, K/R, N/S
	Chloride channel	I/V, K/R, N/S

Table 1. ADAR substrates with editing sites in coding regions.

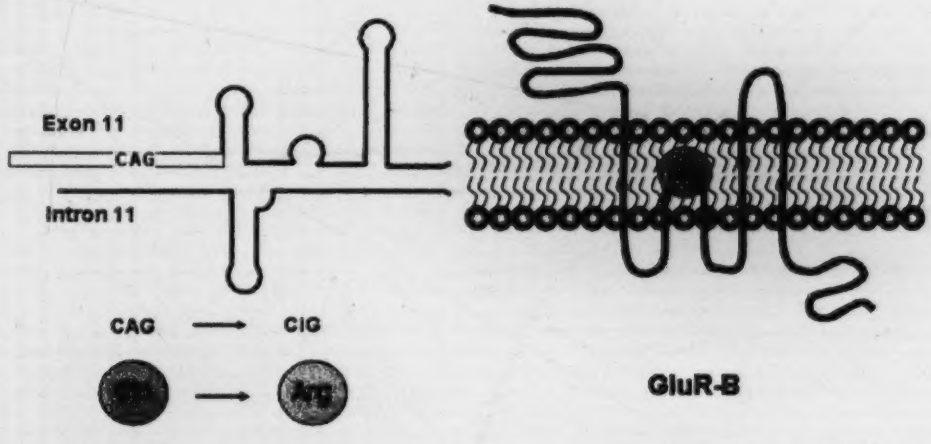

Figure 1-5. Q/R site on GluR-B. The editing changes codon CAG to CIG, which is recognized by ribosome as CGG resulting in the change of glutamine to arginine. The location of the arginine in a model of protein is shown on the right.

expression of up to 24 isoforms with very different efficiency with which the receptors couple to G protein. A study comparing schizophrenic and healthy brain reveals that the abundance of certain isoforms differs significantly in tissue isolated from the frontal cortex (32).

Hepatitis delta virus (HDV)

The genome of HDV is a circular single-stranded RNA of ~ 1700 nucleotides which replicates through an intermediate RNA: the antigenome. Both the genome and antigenome of HDV form covalent closed circles that are predicted to base-pair along the entire length. The ADAR1 targets at a single adenosine in the only expressed HDV open-reading frame, to convert an amber stop codon to a tryptophan (33). This editing results in the generation of one long viral protein, HD-Ag-L, instead of the generation of one short viral protein, HD-Ag-S, from the unedited RNA. Both proteins are required for the viral life cycle: HD-Ag-L for assembly of new viral particles and HD-Ag-S for viral replication.

3.2 Editing in non-coding regions

With the hope of finding more ADAR substrates with editing sites in coding regions, a systematic way to identify inosine was applied to poly A+ RNA isolated from mixed populations of *C. elegans* (34) and rat brain (35) and a global scanning for editing sties has been conducted (36-39). Surprisingly, the results suggest most editing sites exist in non-coding regions such as 5′-UTR, 3′-UTR, Alu repeats and LINE repeats. However, the function of editing at non-coding sites is only partly understood.

Creating splice site

ADAR2 targets its own pre-mRNA at an AA dinucleotide to create an AI site, which is recognized by the splicing machinery as a typical AG 3′ splice site (40). The

creation of a new splice site results in the insertion of a 47-nucleotide fragment inducing a stop codon in mRNA, generating an 88-amino acid polypeptide lacking both dsRBDs and the catalytic domain. This editing is considered as a feedback mechanism that regulates the *in vivo* level of ADAR2 protein. So far, it is the only case in which editing is found to alter splice sites.

Interacting with RNAi pathway

Primary miRNAs (pri-miRNA) are encoded in the human genome and some viral genomes. The pri-miRNA consists of multiple sets of imperfect base-paired short dsRNA hairpins. Drosha and DGCR8 cleave pri-miRNA to release intermediate precursor miRNAs (pre-miRNAs), which are exported into the cytoplasm. After the cleavage of these pre-miRNAs by Dicer and TRBP, duplex RNA is generated, of which both strands of RNA can be used to silence target genes (Figure 1-6). The dsRNA structure of pri-miRNA and pre-miRNA makes them potential targets of ADAR proteins. It has been reported that editing at two specific sites of pri-miRNA-142 completely suppresses the cleavage by Drosha-DGCR8 complex (41) and editing of pri-miRNA-151 blocks the interaction of the Dicer-TRBP complex with the pre-miRNA (42). A recent report reveals that editing on miRNA sequence redirects mature miRNA to novel targets (43).

3.3 Other functions of ADARs

The recent identification of proteins bound to inosine-rich RNA implies more functions of ADAR proteins. Nuclear localized protein, $p54^{nrb}$ can bind to

hyper-edited RNA to retain the RNA in nuclear speckles (44, 45). The identification of Vigilin in a complex formed together with ADAR1, Ku86-Ku70, and RNA helicase A suggests the potential role of ADAR protein in heterochromatin formation and gene silencing at the DNA level (46).

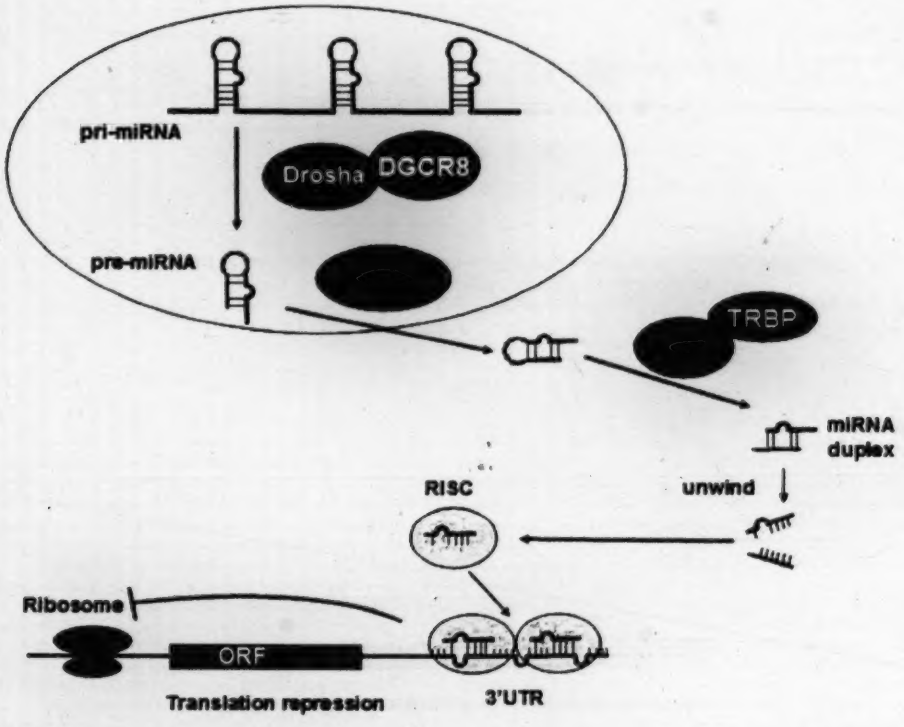

Figure 1-6. RNAi pathway (miRNA pathway). Primary miRNA (pri-miRNA) is cleaved by the complex of Drosha and DGCR8 to form pre-miRNA. pre-miRNA is exported to cytoplasm where it is further cleaved by Dicer and TRBP. The mature miRNA is loaded into RISC to bind to target genes.

4. ADAR SPECIFICITY

ADARs have a wide range of specificities depending on the structure of their substrates. For long perfectly base-paired RNA, adenosines are deaminated nonselectively. For substrates that are longer than 20bp containing mismatches, bulges and loops, ADAR only catalyzes the deamination reaction at one or more specific adenosines. *In vitro* studies show that ADAR proteins have slight sequence preferences for the deaminating sites. *Xenopus* ADAR1 exhibits a preference of U=A>C>G 5′ to the editing site (47) while ADAR2 has a preference of U≈A>C=G 5′ to the editing site and also a preference of U=G>C=A 3′ to the editing site (48). This preference for 5′ and 3′ adjacent nucleotides may be explained by the architecture of active site in the catalytic domain as they may both interact with the pocket which the active site locates in as mentioned above.

The neighbor preference can not thoroughly explain the specificity of ADARs on some substrates. For example, ADAR precisely catalyzes the reaction at the adenosine of the amber stop codon in HDV antigenome among more than 300 adenosines.

The two dsRBDs of rat ADAR2 have been shown to have different binding selectivity on R/G hairpin RNA based on its structure. The editing efficiency at R/G site by ADAR2 decreases dramatically when the two dsRBDs are deleted (16). Together these data suggests that the dsRBD does not only direct ADAR proteins to substrates but also plays important roles in the selectivity of ADARs. However, the catalytic domain has also been shown to play a role in the recognizing of substrates as exchanging of catalytic domains between ADAR1 and ADAR2 affects the editing

efficiency at specific sites (49). The fact that the catalytic domain alone is active *in vitro* although the efficiency is low indicates that this domain also affects selectivity (17). How ADAR acquires specificity seems to be more complicated than previously considered and both dsRBDs and the catalytic domain may be involved.

All the studies on substrate discovery and substrate specificity have been conducted on ADAR1 and ADAR2, while ADAR3 has not been extensively studied because it has been shown that ADAR3 has no catalytic activity on substrates edited by ADAR1 and ADAR2. As ADAR3 has a similar domain composition to ADAR1 and ADAR2 and is the only isoform uniquely expressed in brain, it is interesting and important to identify its substrates and explore its function. In Chapter 2, RT-PCR and PCR experiments conducted to amplify the ADAR3 gene from various sources are described and the expression of tagged ADAR3 protein in mammalian cells is explored. Fluorescence and FRET studies were conducted to study the *in vivo* localization of ADAR3 and the formation of homodimer and heterodimers with ADAR1 and ADAR2. In Chapter 3 of this thesis, ADAR3 deaminase domain and a fusion protein, ADAR3 deaminase domain with N-terminus of ADAR2, were examined for editing activity *in vitro*.

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CHAPTER 2

Cloning, expression and *in vivo* characterization of human ADAR3

Introduction

RNA transcripts are subject to various post-transcriptional processes including RNA editing. There are two types of single nucleotide RNA editing, cytidine to uridine and adenosine to inosine, in both of which a deamination reaction is involved. Adenosine to inosine conversion is the most prevalent editing process and has been intensively studied in the last twenty years.

Inosine is recognized by the ribosome and other processing enzymes as guanosine as it base-pairs with cytidine. The conversion of adenosine to inosine in the coding regions of an mRNA may alter the codons which will diversify the initial information encoded by DNA. Many mRNAs encoding neuronal ion channels and some viral RNAs have been shown to undergo this process. For example, there are two editing sites in the mRNA of the glutamate receptor subunit B (GluR-B), Q/R and R/G sites, of which the Q to R change affects the Ca^{2+} permeability of the channel (1-4). Five editing sites have been identified in the coding region of the mRNA of one subtype of the serotonin receptor, 5-HT_{2C}R (5, 6). The altering of amino acids caused by editing of the mRNA of this receptor results in the expression of up to 24 isoforms which differ in the efficiency with which the receptors bind to G protein. The antigenome of hepatitis delta virus is also a substrate of the editing process in which a UAG stop codon is converted to a UIG tryptophan codon (7). Editing in non-coding regions has been shown to introduce a new splicing site on pre-mRNA of rat ADAR2 (24) and the RNAi pathway (8-10).

ADARs (Adenosine Deaminase that act on RNA) have been shown to catalyze the deamination reaction converting adenosine to inosine. ADAR activity was first described as an unwinding of double-stranded RNA (11-13). This was later found to be

due to non-specific deamination of RNA substrates (14). The first ADAR gene, ADAR1, was identified in both human and rat (15, 16). Subsequently, ADAR genes have been identified in species ranging from invertebrates to vertebrates. Different species possess different numbers of ADAR genes. For example, *C. elegans* has two ADAR genes (17) and *D. melanogaster* has only one ADAR (18). Three ADAR genes have been identified in humans, ADAR1, ADAR2 and ADAR3. The three human ADAR proteins have a similar domain structure that includes multiple double-stranded RNA binding domains (dsRBDs) and a conserved C-terminal catalytic domain (Figure 2-1).

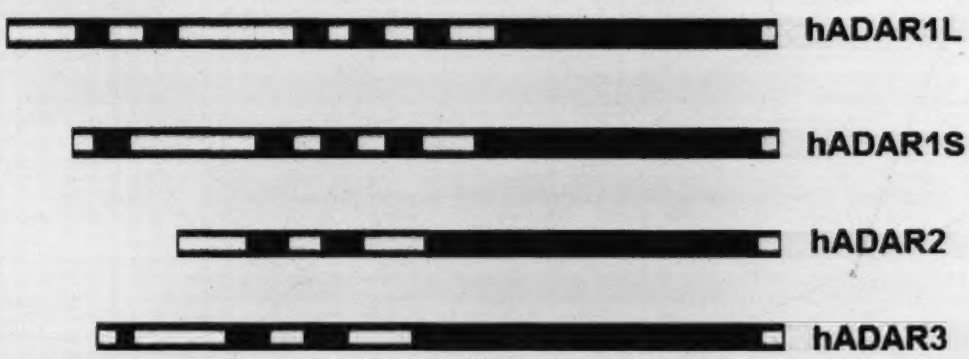

Figure 2-1. Domain structure of human ADAR proteins. Green box: Z-DNA binding domain; Dark blue box: double-stranded RNA binding domain (dsRBD); Red box: arginine-rich domain; Light blue box: deaminase domain; Yellow region: linker.

Among the three human ADAR proteins, ADAR1 and ADAR2 are the best characterized. There are two isoforms of ADAR1. A long isoform, ADAR1L, is transcribed from an interferon-inducible promoter on the *ADAR1* gene. ADAR1L has a cytoplasmic as well as nuclear localization and may be involved in response to viral infection (19, 20). A constitutive short nuclear isoform, ADAR1S, is transcribed from a downstream promoter leading to a truncation of the N-terminal region. ADAR1 has been shown to efficiently edit the R/G site of GluR-B mRNA and the antigenome of HDV but

has a low deaminase activity on a number of specific substrates including the Q/R site of GluR-B mRNA (7, 21-23). ADAR2 has been shown to efficiently edit both the R/G and Q/R sites of GluR-B mRNA and also its own mRNA which creates a new splicing site resulting in the generation of an inactive truncated peptide (24).

ADAR3 is the most recent ADAR gene to be identified among the three human ADARs. A new ADAR gene of rat, RED2, was cloned in 1996 (25); the corresponding protein was found only in brain tissue. A human homolog of this gene, ADAR3, was identified by Nishikura and her colleagues (26). This new human ADAR protein was also shown to be only expressed in brain, especially in particular regions such as the amygdala and thalamus. This is in contrast to ADAR1 and ADAR2, which are expressed in many tissues other than the brain. ADAR3, as shown in Figure 2-1, has an extra N-terminal arginine-rich domain, which was shown to have affinity for both single-stranded RNA and double-stranded RNA. Although ADAR3 has a very similar domain structure to ADAR2 and conserved catalytic motifs, no editing of known RNA substrates by recombinant human ADAR3 purified from insect cells has been demonstrated and no substrates of ADAR3 have ever been identified (26). The activity of ADAR3 may depend on some specific factors found in human cells. Given the fact that a recent global scanning for editing sites revealed that more editing sites exist than previously predicted (27-32), it is interesting and important to explore the function and physiological role of human ADAR3. We performed RT-PCR and PCR experiments to amplify the ADAR3 gene from various sources and attempted to express the protein in human cells to examine the activity and co-factors of this protein.

As mentioned in Chapter 1, cytidines in RNA transcripts are also subject to deamination which converts cytidines to uridines. This conversion is catalyzed by cytidine deaminases (CDAs). The CDA family has many members, such as the apolipoprotein B mRNA-editing enzyme catalytic polypeptide proteins (APOBEC) and *E. coli* cytidine deaminase (ECCDA). The composition of the catalytic center of cytidine deaminase is the same as ADARs, comprising one glutamate, one histidine and two cysteins coordinating a zinc ion. Thus, CDAs is considered to be the homolog of ADARs. The crystal structure of ECCDA has shown that the protein acts as a dimer and both monomers contribut to the active site (37). The question arises, does ADAR act as a dimer and is the dimerization RNA-independent or RNA-dependent? These questions have been addressed using different methods. ADAR2 was shown to require the formation of a homodimer to efficiently edit substrates when the *in vitro* editing of the GluR-B R/G site was examined, and the formation of this dimer was originally concluded to be RNA-dependent (33). An experiment carried out by purifying recombinant, tagged human ADAR1 and ADAR2 showed that both of the two proteins can form homodimers but not heterodimers and the formation of the homodimers was RNA-independent (34). *In vivo* fluorescent resonance energy transfer (FRET) studies with human ADAR1 and ADAR2 from insect cells confirmed that the dimerization of ADAR1 and ADAR2 was RNA-independent. However, the two proteins were also shown to form heterodimers (35), which suggested that ADARs could expand their range of substrates by forming heterodimers. One hypothesis about ADAR3's potential function is that it may compete with ADAR1 and ADAR2 for binding to substrates and thus inhibit the editing by ADAR1 and ADAR2 (26). It is also possible that ADAR3 may form heterodimers with

ADAR1 and ADAR2 and catalyze editing on unknown substrates as ADAR3 has been shown to be able to bind to single-stranded RNAs but no editing activity on known substrates of ADAR1 and ADAR2 has been demonstrated. To verify this hypothesis, the cyan and yellow fluorescent proteins (CFP and YFP) were used to tag human ADAR3 at the N-terminus and FRET experiments were conducted.

EXPERIMENTAL METHODS

Oligonucleotide Synthesis

All oligonucleotides were purchased from the University of Alberta DNA Core Services Laboratory.

PCR

PCR reactions were performed using Platinum® Pfx DNA Polymerase (Invitrogen) in a 50 µl reaction mix containing 1 X Pfx amplification buffer, 1 mM $MgCl_2$, 10% DMSO, 1 X PCR enhancer, 200 µM of each dNTP, 500 nM of each primer. The thermal cycle used was as follows: (1) 3 min at 94°C, (2) 30 sec at 94°C, (3) 30 sec at 55°C, (4) 2.5 min at 68°C, (5) 30 sec at 94°C, (6) 30 sec at 54°C, (7) 2.5 min at 68°C, (8) 30 sec at 94°C, (9) 30 sec at 53°C, (10) 2.5 min at 68°C, (11) 30 sec at 94°C, (12) 30 sec at 52°C, (13) 2.5 min at 68°C, (15) 30 sec at 94°C, (16) 30 sec at 51°C, (17) 2.5 min at 68°C, (18) 30 sec at 94°C, (19) 30 sec at 50°C, (20) 2.5 min at 68°C, (21) go back to step (18) and repeat for 30 cycles, (22) 10 min at 68°C, (23) End cycle.

RT-PCR

Total RNA extracts from human brain tissue (Alberta Tumor Bank), HeLa cells, HEK 293 cells and SH-SY5Y cells were used as templates to synthesize cDNA. 3 μg of total RNA extract was used in each reaction. Reactions were performed using the ThermoScript™ RT-PCR System (Invitrogen). PCR reactions using synthesized cDNAs as templates were performed using the cycles described above.

DNA extraction

Human HeLa cells were plated in 10 cm tissue culture dishes and allowed to adhere and grow up to 90% confluence. Cells were then collected and centrifuged for 10 min at 10°C (1200 rpm). The supernatant was removed and the cell pellet was resuspended and washed in 10 ml 1 X PBS twice, and centrifuged between washes. The pellet was then resuspended in 10 ml DNA buffer (200 mM Tris-HCl, 100 mM EDTA, pH 8.0) and centrifuged for 10 min at 10°C (1200 rpm). The pellet was saved, resuspended in 3 ml DNA buffer with 125 μl Protease K (10 mg/ml) and 400 μl 10% SDS, and incubated overnight at 45°C. Phenol (3.6 ml) was added to the solution which was mixed by shaking by hand for 10 min at room temperature. The solution was then centrifuged for 10 min at 10°C (3000 rpm). The supernatant was transferred into a new tube (15 ml). An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added to the supernatant. The mixture then was shaken for 10 min at room temperature and centrifuged for 10 min at 10°C (3000 rpm). The supernatant was transferred to a new tube and a 1/10 volume 3 M sodium acetate (pH 5.2) and 3 volumes 100% isopropanol were added to the supernatant. The mixture was shaken gently until the DNA precipitated. The

precipitated DNA was then transferred into a tube with 30 ml 70% ethanol using a sterile glass pipette. The tube was then placed on an inverting rack and inverted for 2 hours until the DNA was thoroughly rinsed. The DNA was then transferred to a sterile eppendorf tube and centrifuged for 20 min at 14,000 rpm. The pellet of DNA was then dried in a SpeedVac for 5 min and dissolved in 500 µl sterile water by shaking overnight at 37°C. The concentration of the extracted DNA was measured using a HP 8451A Diode Array Spectrophotometer.

Total RNA extraction

Total RNA was extracted using the RNAspin Mini RNA isolation kit (GE Healthcare) following the manufacturer's protocol. The concentration of extracted total RNA was measured using a HP 8451A Diode Array Spectrophotometer. The purity and integrity of extracted total RNA were determined by measuring the ratio of absorbance at 260 and 280 nm and running a denaturing agarose gel.

Mammalian expression constructs

DNA encoding human ADAR3 was amplified by PCR and cloned into expression vectors and CFP/YFP fusion vectors as below:

Vector	Primers used	Restriction sites
pECFP-C1 (Clonetech)	Forward: 5′-GCGCAGATCTATGGCCTCGGTCCTGG-3′ Reverse: 5′-GCGCGCAAGCTTCTAGAGAGTCAGTAGAAAC-3′	Bgl II, Hind III

pEYFP-C1 (Clonetech)	Forward: 5′-GCGCAGATCTATGGCCTCGGTCCTGG-3′ Reverse: 5′-GCGCGCAAGCTTCTAGAGAGTCAGTAGAAAC-3′	Bgl II, Hind III
p3XFLAG-CMV™-10 (Sigma)	Forward: 5′-GCTAAAGCTTATGGCCTCGGTCCTG-3′ Reverse: 5′-GCGCGTTCTAGACTAGAGAGTCAGTAGAAACTGC-3′	Hind III, Xba I

Cell culture and transfection

Human HeLa cells, HEK 293 cells and SH-SY5Y cells were used for protein expression and fluorescent experiments. HeLa and HEK 293 cells were cultured as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS at 37°C and 5% CO_2. SH-SY5Y cells were cultured in a mix of 50% DMEM and 50% F12 medium with 10% FBS at 37° C and 5% CO_2.

For fluorescence experiments, cells (2 x 10^5) were plated on 25 mm coverslips in six-well tissue culture dishes and allowed to adhere for 24 hours. The cells were then transiently transfected with 1 μg plasmid using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol and analyzed 24 hours post-transfection.

To create stable cell lines expressing ADAR3, cells were plated and transfected as described above. G418 disulfate salt solution (200 μl) (50 mg/ml) was added into the medium 24 hours post-transfection. The medium containing G418 was changed every 3-4 days for two weeks. Single colonies of cells were plated in 10 cm tissue culture dishes separately to examine the expression level of ADAR3.

Protein expression and purification

ADAR3 was cloned into the p3XFLAG-CMV™-10 vector for over-expression in human cells. Cells were plated in 10 cm tissue culture dishes and transiently transfected with 8 μg plasmid DNA using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol and harvested 24 hours post-transfection. Cells were incubated in 500 μl lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, pH 7.4) for 30 min and centrifuged for 10 min at 150,000 g. The supernatant was then incubated with 20 μl ANTI-FLAG® M2 affinity gel (Invitrogen) (prepared according to the manufacturer's protocol) for 2 hours and centrifuged for 30 sec at 8,000 g. The supernatant was discarded and the tagged protein was then eluted by incubating the gel with 40 μl wash buffer (50 mM Tris, 200 mM NaCl, pH 7.4) with 150 ng/μl 3 X FLAG peptide for 30 min and centrifuged for 1 min at 8,000 g.

Protein isolation with TRIZOL® Reagent

Human HeLa, HEK 293 and SH SY5Y cells (2×10^5) were plated in six-well tissue culture dishes and allowed to adhere for 24 hours. The cells were then transiently transfected with 1 μg plasmid using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's protocol and analyzed 24 hours post-transfection.

TRIZOL® reagent of 1 ml was added to each well and then transferred into a 1.5 ml eppendorf tube. The solution was then mixed well with 0.2 ml chloroform and incubated at room temperature for 2-3 min and centrifuged at 120,000 g for 8 min at 4°C. The lower phase was then transferred to a new 1.5 ml eppendorf tube and mixed well with 0.3 ml 100% ethanol, incubated at room temperature for 2-3 min and centrifuged at 2,000 g for 5

min. The supernatant was transferred to a new 2 ml eppendorf tube and mixed with 1.5 ml isopropanol, incubated at room temperature for 10 min and centrifuged at 120,000 g for 10 mins at 4°C. The pellet was then rinsed in 1 ml 95% ethanol with 0.3 M GuHCl and incubated at room temperature for 20 min and centrifuged at 7,500 g at 4°C for 5 min. The pellet was washed in the same solution twice more and then washed in 1 ml 100% ethanol and centrifuged at 7,500 g at room temperature. The pellet was then dried by vacuum and resuspended in 100 µl ddH_2O with 1% SDS and shaken at 37 °C overnight to dissolve.

The concentration of isolated protein was determined by using Biorad Protein Assay. 10 µg of total protein was loaded for SDS-PAGE and Western blot.

Western blot

A Western blot was performed after gel electrophoresis. The proteins were transferred to nitrocellulose or PVDF membranes using a transfer buffer (3.03 g Tris, 14.4 g glycine, 200 ml methanol, 0.05% SDS in 1 L ddH_2O). The membrane was then washed twice (10 min each) in 20 ml 1 X TBST buffer (20 mM Tris, 137 mM NaCl, 0.1 % Triton X-100, pH 7.6) at room temperature and blocked in 50 ml 1 X TBST with 5% milk powder at 4°C overnight. The membrane was washed in 20 ml 1 X TBST buffer three times (10 min each). For Flag-tagged protein detection, the membrane was probed in 10 ml 1 X TBST with 1:1000 diluted Monoclonal ANTI-FLAG® M2-Peroxidase (HRP) (Sigma) for 1 hour at room temperature. The membrane was then washed twice in 1 X TBST (5 min each) and developed using commercial detection reagents (Amersham).

Fluorescence microscopy

Transiently transfected cells which were seeded on coverslips were washed 3 times in PBS and fixed for 15 min in freshly made 4% formaldehyde (Sigma) at room temperature. The coverslips were washed 3 times in PBS and then mounted on slides using DAPI solution (90% glycerol, 10% PBS and 1μg/ml DAPI). Images were collected with a Zeiss Laser Scanning Confocal Microscope (LSM 510 NLO Meat). The 514 and 458 nm laser lines were used to image YFP and CFP. Band pass filters of 580-612 nm and 462-484 nm were used in collecting emission from YFP and CFP respectively.

Fluorescence resonance energy transfer

Transiently transfected cells which were seeded on coverslips were washed 3 times in PBS and fixed for 15 min in freshly made 4% formaldehyde (Sigma) at room temperature. The coverslips were washed 3 times in PBS and then mounted on slides using a mounting solution (90% glycerol, 10% PBS). FRET experiments were conducted using the donor recovery after acceptor photobleach method (36). The images were obtained in the CFP and YFP channels first and the intensity of each signal was calculated. YFP was then photobleached to 20% of its original fluorescence at 514 nm in a defined region. A second set of images was taken after the photobleaching as before and intensity was calculated. FRET efficiency was calculated as below:

$$\text{FRET efficiency} = (D_{post} - B_{post}) - (D_{pre} - B_{pre}) / (D_{post} - B_{post})$$

where D is the donor channel (CFP) intensity, B is the background intensity, and pre and post refer to measurements made before and after photobleaching. A non-bleached region of the same cell was used as an internal control.

RESULTS

1. Cloning of human ADAR3 gene

1.1 Cloning of human ADAR3 gene from human amygdala cDNA

As human ADAR3 was demonstrated to be enriched in the amygdala and thalamus (26), we hoped to amplify full-length ADAR3 gene from human amygdala cDNA (USBiological), which failed. The cloning strategy was then changed to divide the ADAR3 gene into two parts, the N-terminus and C-terminus, and clone them separately. We succeeded in cloning the C-terminus but not N-terminus (Figure 2-2).

1.2 Cloning of human ADAR3 gene from total RNA of HeLa and HEK 293 cells

As shown in Figure 2-3, we divided ADAR3 N-terminus into different fragments and tried to amplify these fragments from total RNA of HeLa and HEK 293 cells but none of the fragments were successfully amplified.

1.3 Cloning of human ADAR3 gene from human chromosome and IMAGE human cDNA clone

Human ADAR3 has been shown to be located on chromosome 16q and the N-terminal fragment of ADAR3 is composed of three exons. As shown in Figure 2-4, fragment 1-279 (fragment 1) was amplified from an IMAGE human cDNA clone (Clone ID 2296958) and fragment 279-1084 (fragment 2) was amplified from genomic DNA extracted from HeLa cells. Fragment 1 was first cloned into the pCR®4-TOPO vector

(Invitrogen), and then cut out with BamH I and EcoR I and cloned into the pGEX 6p1 vector (GE Healthcare) using the same restriction enzymes. Fragment 2 was cloned into pGEX 6p1 with fragment 1 cut by Asc I and EcoR I. The ADAR3 C-terminus was subsequently cloned into the this vector using EcoR I and Xba I (Figure 2-5). The sequence of the full-length ADAR3 gene was verified by plasmid sequencing.

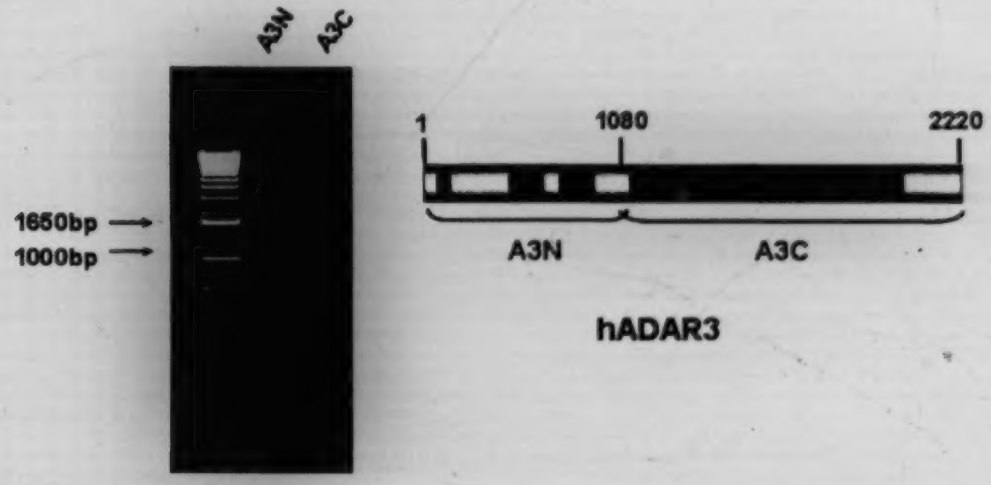

Figure 2-2. Cloning of ADAR3 N and C terminus from human thalamus cDNA. Left: 1% agarose gel of PCR products of ADAR3 N and C terminus from human thalamus cDNA. 1 Kb Plus DNA Ladder (Invitrogen) was used as a marker. Right: ADAR3 N-terminus and C-terminus. The numbers above refer to nucleotide numbers corresponding to the beginning and end of two fragments.

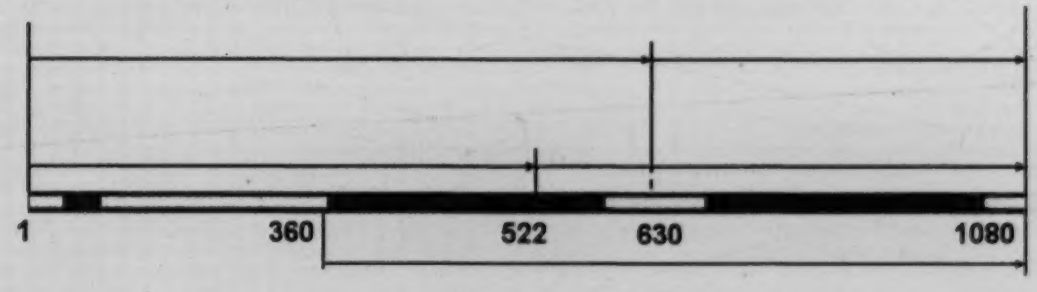

Figure 2-3. Dividing the ADAR3 N-terminus into different fragments for cloning. The numbers refer to nucleotide numbers corresponding to the beginning and end of indicated fragments.

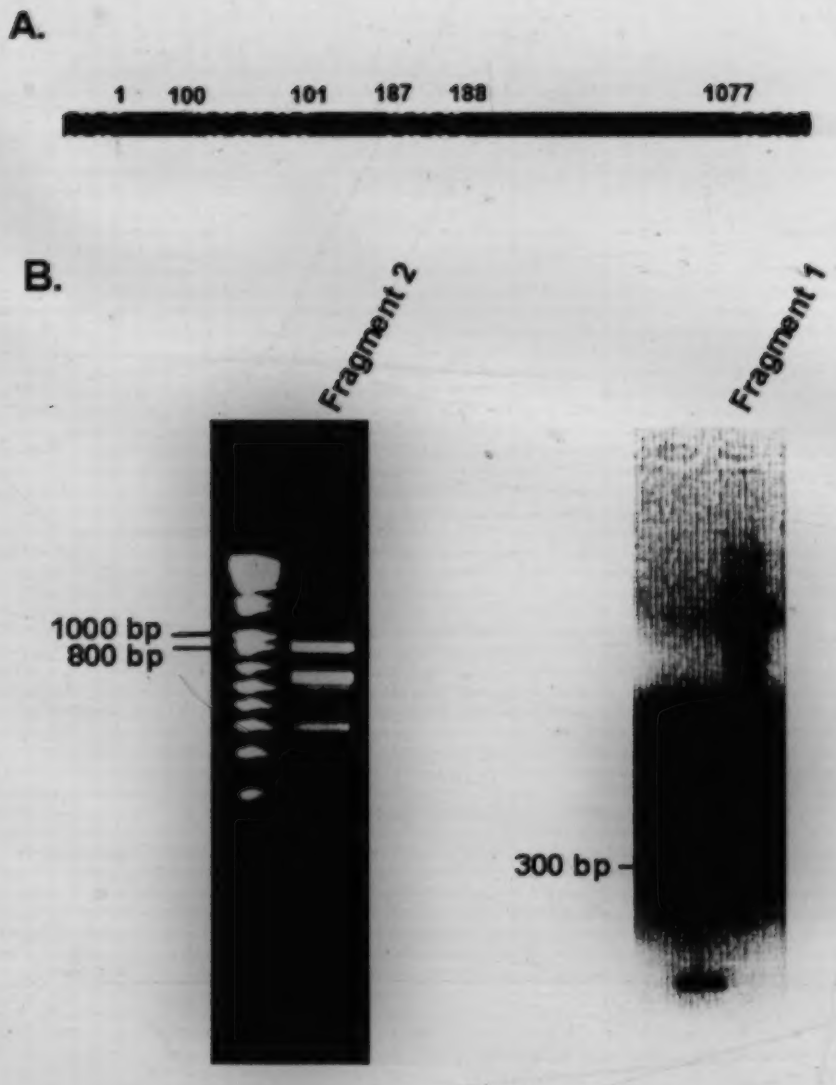

Figure 2-4. Cloning of two fragments of ADAR3. A. Exons of human ADAR3 N-terminus in chromosome 16. ADAR3 N-terminus consists of three exons located on chromosome 16. The red boxes indicate exons and blue ones indicate introns and 5′ untranslated region. The numbers above are nucleotide numbers of ADAR3 mRNA. B. Left: Cloning of fragment 2 (279-1084) of ADAR3 N-terminus from chromosome. 1 Kb Plus DNA Ladder (Invitrogen) was used as a marker. Right: Cloning of fragment 1 (1-279) of ADAR3 N-terminus from IMAGE clone of human cDNA. O'GeneRuler™ 100 bp DNA Ladder (Fermentas) was used as a marker. Red boxes indicate the fragments of the appropriate size.

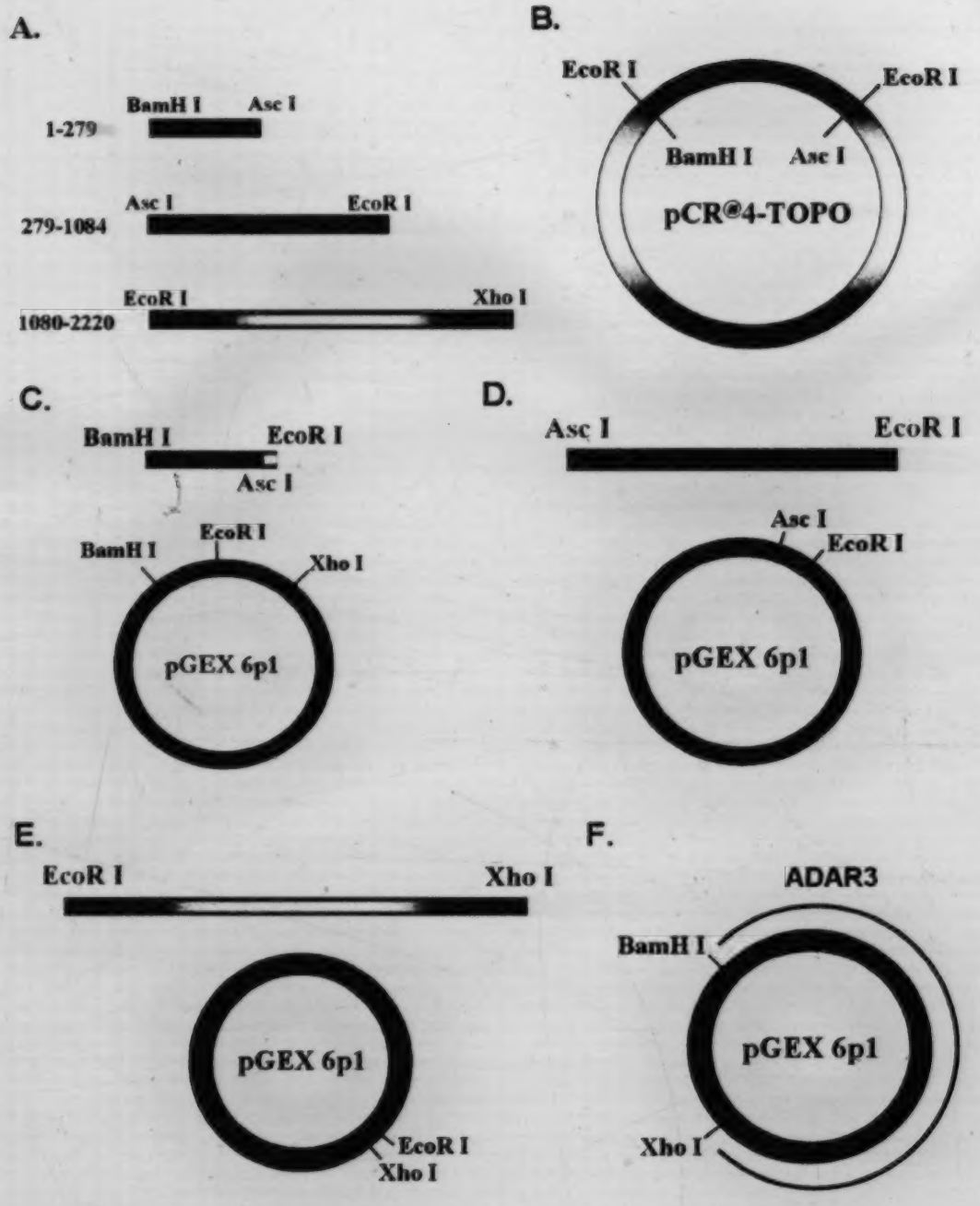

Figure 2-5. Strategy to merge ADAR3 fragments together. Red: intrinsic restriction enzyme sites on ADAR3 gene. Black: introduced restriction enzyme sites. A. Three fragments of ADAR3 are shown with nucleotide number shown on the left. B. fragment 1 is cloned into pCR@4-TOPO vector. C. fragment 1 is cut off pCR@4-TOPO vector and cloned into pGEX 6p1 vector. D. fragment 2 is cloned into pGEX 6p1 vector with fragment 1 cloned in. E: fragment 3 is cloned into pGEX 6p1 vector with fragment1 and 2 cloned in. F: Full-length ADAR3 gene is cloned into pGEX 6p1 vecotr.

Figure 2-6. RT-PCR from SH SY5Y and HEK 293 cells. A. RT-PCR of ADAR3 C-terminus (~1120 bp) from total RNA extracted from HEK 293 and SH SY5Y cells. Lanes 1-6 are RT-PCR performed under different conditions using total RNA from HEK 293 cells and lanes 7-12 are RT-PCR performed under the same condition changing the template to total RNA from SH SY5Y cells. Red box shows the expected product. B. Right lane: RT-PCR of full-length ADAR3 gene (2220 bp) from total RNA extracted from SH SY5Y cells. O'GeneRuler™ Express DNA Ladder (Fermentas) was used as a marker.

1.4 Cloning of human ADAR3 gene from total RNA of SH-SY5Y cells

ADAR3 has previously been reported to be only detected in postmitotic neurons (26). To confirm the transcription of full length ADAR3 mRNA, we used total RNA extract from SH-SY5Y cells, a third generation of neuroblastoma, as template to perform RT-PCR. As shown in Figure 2-6, both the ADAR3 C-terminus and full-length ADAR3 were amplified by RT-PCR from total RNA extract of SH SY5Y cells but not from HEK 293 cells. Sequencing revealed a mutation occurring on the RT-PCR product that cysteine at 490 was mutated to tyrosine.

2. *Over-expression of human ADAR3*

2.1 Over-expression of human ADAR3 in HEK 293 and SH-SY5Y cells

ADAR3 was cloned into the p3XFLAG-CMV™-10 vector for over-expression in human cell lines. Transient transfection of ADAR3 was conducted with HeLa, HEK 293 and SH SY5Y cells. Total protein was isolated using TRIZOL® reagent and Western blot was then performed to detect the expression of ADAR3 protein (Figure 2-7).

Figure 2-7. Expression of ADAR3 in HeLa, HEK 293 cells and SH SY5Y cells. Lane 1: ADAR2 over-expressed in HEK 293 cells using p3XFLAG-CMV™-10 vector. Lane 2-4: ADAR3 over-expressed using p3XFLAG-CMV™-10 vector in HEK 293, SH SY5Y and HeLa cells respectively. The expression of full-length ADAR3 can be detected in HeLa and HEK 293 cells with degradation. In SH SY5Y cell, ADAR3 was totally degraded.

3. *Cellular localization of human ADAR3*

Human ADAR1 and ADAR2 have been shown to localize predominantly in the nucleolus (35). We wanted to determine the localization of ADAR3 in living cells. ADAR3 was cloned into the pECFP-C1 vector used for transient transfection of human HEK 293 cells. Expression of CFP-ADAR3 was at almost the same level in experiments

involving CFP-ADAR1 or CFP-ADAR2. ADAR3 exhibited a predominantly nucleolar localization similar to ADAR1 and ADAR2 (Figure 2-8).

4. Homo and heterodimerization of ADAR3 and ADAR1, ADAR2

Human ADAR1 and ADAR2 have been shown to be able to form not only homodimers but also heterodimers with each other *in vivo* by FRET (35). Given the similarity of domain structure among the three human ADARs, we wanted to determine whether ADAR3 can form either homo or heterodimers with the other two active enzymes. ADAR3 was cloned into the pECFP-C1 and pEYFP-C1 vectors encoding N-terminal CFP and YFP respectively. We prepared a combination of vectors used for co-transfection and FRET experiments as follows: CFP-ADAR3/YFP-ADAR3, CFP-ADAR3/YFP-ADAR1 and CFP-ADAR3/YFP-ADAR2. We observed 19.2±3.2% FRET efficiency for CFP-ADAR3/YFP-ADAR3 (Figure 2-9) compared to the FRET efficiency of 21.3±1.2% measured in an experiment using tandem CFP-YFP joined by a short ten amino acid linker (data not shown). A FRET efficiency of 16.5±1.0% and 17.3±1.9% were measured for CFP-ADAR3/YFP-ADAR1 (Figure 2-10 A) and CFP-ADAR3/YFP-ADAR2 (Figure 2-10 B) respectively. Compared with the data from FRET experiments on ADAR1 and ADAR2 dimerization, our data strongly suggested that ADAR3 forms homodimer as well as heterodimers with both ADAR1 and ADAR2.

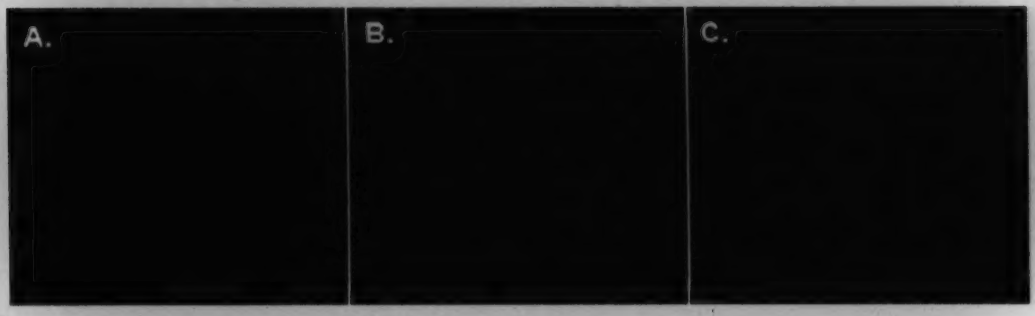

Figure 2-8. *In vivo* localization of ADAR3. A. DAPI image of HeLa nuclei. B. Confocal fluorescence imaging of CFP-ADAR3 in transfected HeLa cells. C. Merged image shows that ADAR3 localizing to the regions of faint DAPI signal which correspond to nucleoli.

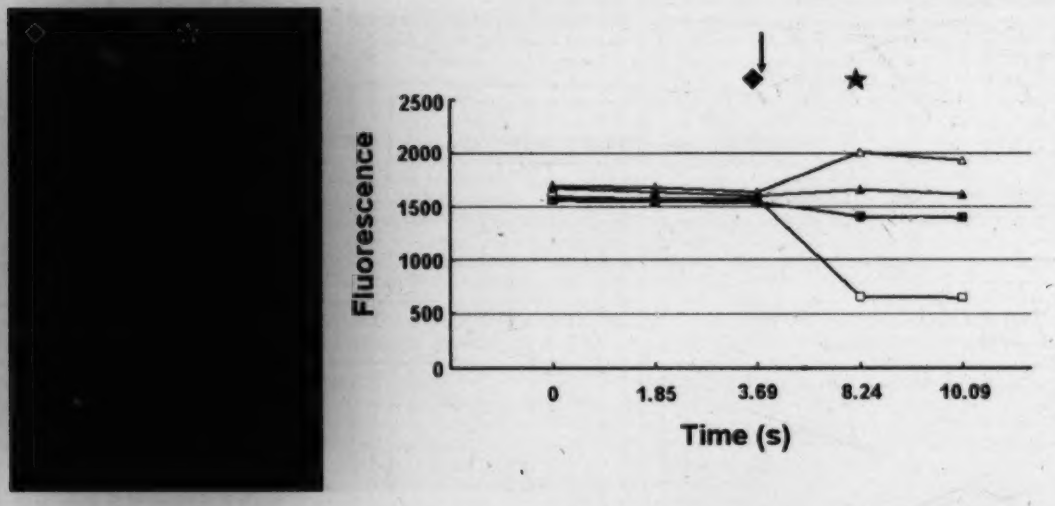

Figure 2-9. Homodimerization of ADAR3. A. Confocal fluorescence imaging of HeLa cells co-transfected with CFP-ADAR3 and YFP-ADAR3. Direct excitation of CFP was performed using a 458 nm laser line. Emissions of CFP filtered at 463-484 nm (top panels), emissions of YFP filtered at 570-612 nm (middle panels) and merge (bottom panels) are shown. Images were taken before (left panels) and after (right panels) specifically photobleaching YFP within a nucleolus of the cell. B. Quantification of CFP and YFP signals of images taken before and after photobleaching (indicated by the arrow). CFP fluorescence is shown for the photobleached nucleolus (open triangles) and a non-photobleached nucleolus (closed triangles). YFP fluorescence is shown for the photobleached nucleolus (open squares) and a non-photobleached nucleolus (closed squares). Quantifications corresponding to images at left are indicated (◆, ★).

DISCUSSION

Although ADAR3 shares a similar domain structure and possesses the conserved catalytic motifs of ADAR1 and ADAR2, it seems to be a very special member of the human ADAR family. ADAR3 is the only family member in which expression is restricted to the brain and enriched in certain regions. It has been shown to have no catalytic activity on known substrates of ADAR1 and ADAR2, although it can bind to these substrates and inhibit the activity of the other two enzymes. The demonstration that ADAR3 protein is only detected in postmitotic neurons and the fact that most RNA substrates of ADARs are neural-specific RNA indicate that ADAR3 may act on some specific substrates in neuron cells and this function may require specific co-factors in neuron cells.

Nothing has been reported with respect to human ADAR3 since the initial paper describing the protein published in 2000 (26). Our experience with cloning of ADAR3 may explain why so little work has been done with ADAR3. The difficulty amplifying mRNA of ADAR3 is either a reflection of low/tissue specific expression or instability of the message. The degradation of ADAR3 in all three human cell lines we have, HeLa, HEK 293 and SH-SY5Y, which were good for the over-expression of ADAR1 and ADAR2, indicated the instability of this protein probably because of its toxicity to cells. Toxicity of ADAR3 could be due to an ADAR3 editing activity or competition of ADAR3 (either alone or in heterodimer combinations) for endogenous ADAR1/ADAR2 substrates thus inhibiting required editing activity. ADAR3 has been shown to be able to compete with ADAR1 and ADAR2 for binding to substrates and thus regulate the editing.

The FRET experiments reported here have shown for the first time that ADAR3 can form homodimers *in vivo*, similar to ADAR1 and ADAR2 and also heterodimers with ADAR1 and ADAR2. The formation of heterodimers among different members of ADAR family has been suggested as a mechanism of expanding/modulating editing activity of ADAR family members. The formation of heterodimers of ADAR3 with ADAR1 and ADAR2 suggests that ADAR 1 and ADAR2 might edit unknown double-stranded RNA substrates or even single-stranded RNA substrates. Although ADAR3 has been shown to be inactive on known substrates, the results reported here suggest that the activity of heterodimers of ADAR3 with ADAR1 and ADAR2 should be examined in the future.

ADAR3 has a similar domain structure to ADAR2 but ADAR3 has an extra N-terminal arginine-rich domain, which is suggested to bind to single-stranded RNA. The deletion of the arginine-rich domain did not make rat ADAR3 active on known substrates (25). Although the three members of human ADAR family share conserved catalytic motifs, the difference in regions beyond the motifs may result in different tertiary structure that affects the activity, At this point, a high resolution structure of full-length ADAR3 or the catalytic domain of ADAR3 will help to explain why ADAR3 is so special.

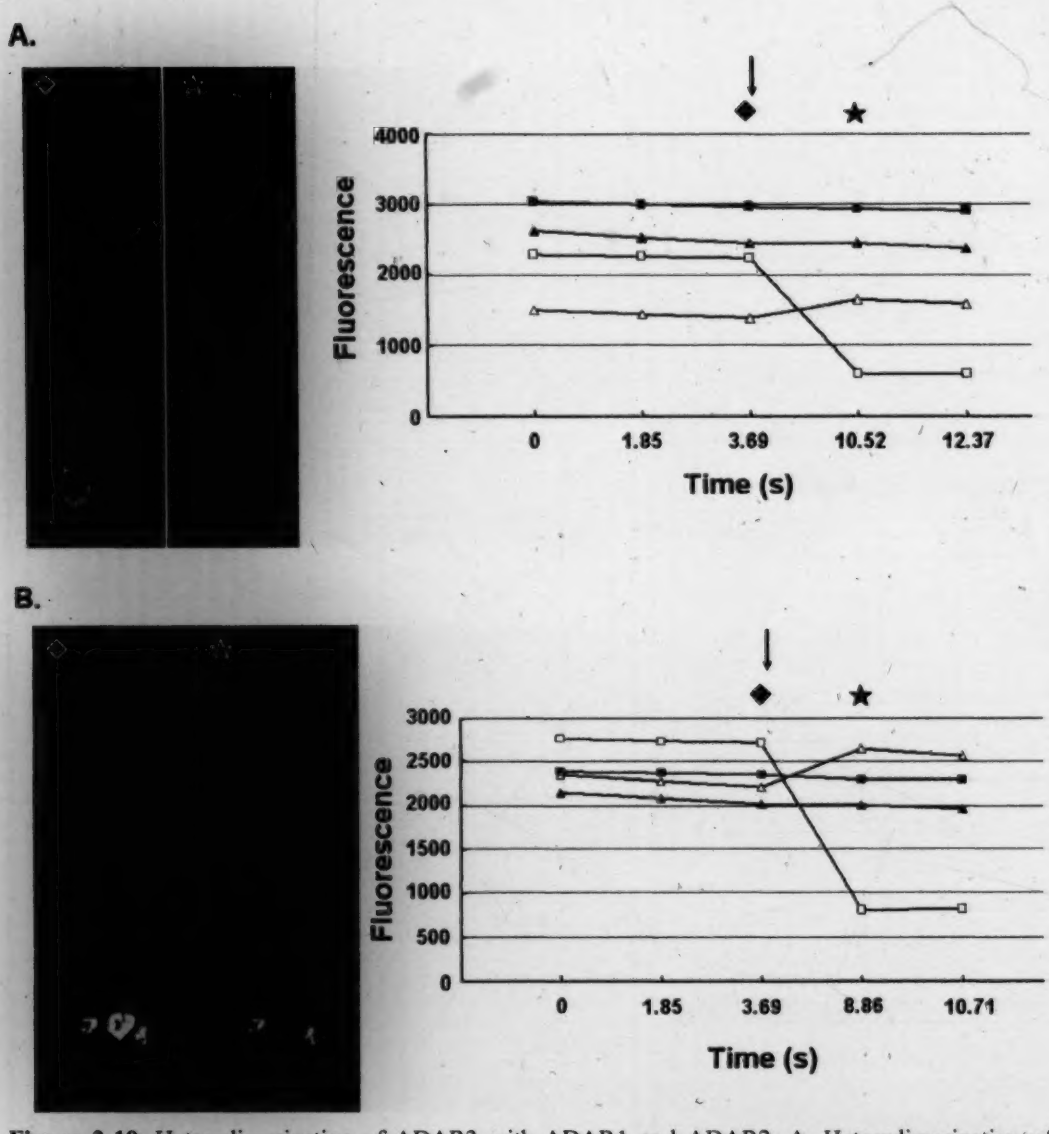

Figure 2-10. Heterodimerization of ADAR3 with ADAR1 and ADAR2. A. Heterodimerization of ADAR3 and ADAR1. Confocal fluorescence imaging of HeLa cells co-transfected with CFP-ADAR3 and YFP-ADAR1. Direct excitation of CFP was performed using a 458 nm laser line. Emissions of CFP filtered at 463-484 nm (top panels), emissions of YFP filtered at 570-612 nm (middle panels) and merge (bottom panels) are shown. Images were taken before (left panels) and after (right panels) specifically photobleaching YFP within a nucleolus of the cell. Quantification of CFP and YFP signals of images taken before and after photobleaching (indicated by the arrow). CFP fluorescence is shown for the photobleached nucleolus (open triangles) and a non-photobleached nucleolus (closed triangles). YFP fluorescence is shown for the photobleached nucleolus (open squares) and a non-photobleached nucleolus (closed squares). Quantifications corresponding to images at left are indicated (◆, ★). B. Heterodimerization of ADAR3 and ADAR2. Confocal fluorescence imaging of HeLa cells co-transfected with CFP-ADAR3 and YFP-ADAR2. The images and quantification were done as the same of the experiment of ADAR1 and ADAR3.

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CHAPTER 3

In vitro Study on activity of human ADAR3 deaminase domain

This work was done collaborating with Edyta Sieminska.

INTRODUCTION

RNA transcripts undergo various maturation processes including 5′ capping, 3′ processing and polyadenylation, splicing and editing. Deamination reactions on either adenosine or cytidine are involved in the editing process. Particular adenosine (A) residues in RNA transcripts are deaminated to be converted to inosine (I), which are recognized by the ribosome and other processing enzymes as guanosine (G). Such A-to-I conversions occurring in the coding regions of RNA transcripts can alter the original coding information of DNA, for example, the editing occurring on the glutamate receptor subunit B pre-mRNA changes a glutamine (Q) to an arginine (R), which is located in the loop forming the channel pore (1, 2) and editing processes on the pre-mRNA of one subtype of serotonin receptor, 5-HT_{2C}R, alter multiple codons (3, 4).

An enzyme family, ADAR (Adenosine Deaminase that act on RNA), has been shown to catalyze the A-to-I deamination reaction. ADAR activity was first discovered in *Xenopus laevis* (5-7) and since then different ADAR proteins have been cloned and characterized in many metazoa including mammals (8-12). Three ADAR genes have been identified in humans, ADAR1 (13), ADAR2 (14-16), and ADAR3 (17). All of the three human ADAR proteins have a similar domain structure, two or three double-stranded RNA binding domains (dsRBD) at the N-terminus and a deaminase domain, at the C-terminus (Figure 3-1).

The C-terminal deaminase domain is the catalytic domain of ADAR proteins and is conserved among ADARs. The X-ray structure at 1.7 Å resolution of the deaminase

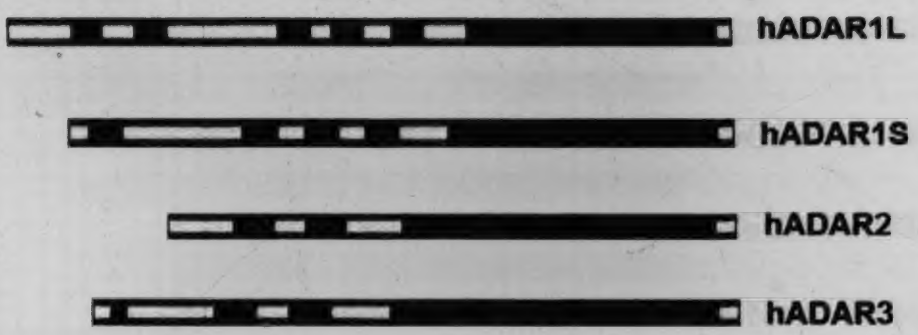

Figure 3-1. Domain structure of human ADAR proteins. Green box: Z-DNA binding domain; Dark blue box: double-stranded RNA binding domain (dsRBD); Red box: arginine-rich domain; Light blue box: deaminase domain; Yellow region: linker.

domain of human ADAR2 has been reported (Figure 3-2A) (18). The catalytic domain adopts a roughly spherical structure ~ 40 Å in diameter. The active site is indicated by a zinc ion coordinated by H394, C451, C516 and a water molecule bound to E396 by hydrogen bonding (Figure 3-2B). The bound water molecule is thought to displace ammonia during the deamination reaction. The location of the active site is in a deep pocket and surrounded by positive electrostatic potential, which probably serves as a binding site of RNA substrates. This deep location of the active site is consistent with the idea that ADARs may use a base-flipping mechanism to expose the C6 of the adenosine for reaction. Interestingly, an IP_6 molecule is found to be buried deep in an extremely basic cavity formed by many arginine and lysine residues at one side of the structure. IP_6 is an abundant inositol polyphosphate involved in multiple cellular functions including RNA export, DNA repair, chromatin remodeling and endocytosis (19-23) and it is reported to affect AMPA receptors, the coding mRNAs of which are substrates of ADAR2 (24). It has been shown that IP_6 is required for ADAR2 activity

and is buried in the cavity during the folding of the domain. Thus, IP_6 may play an important role in the folding process of the catalytic domain.

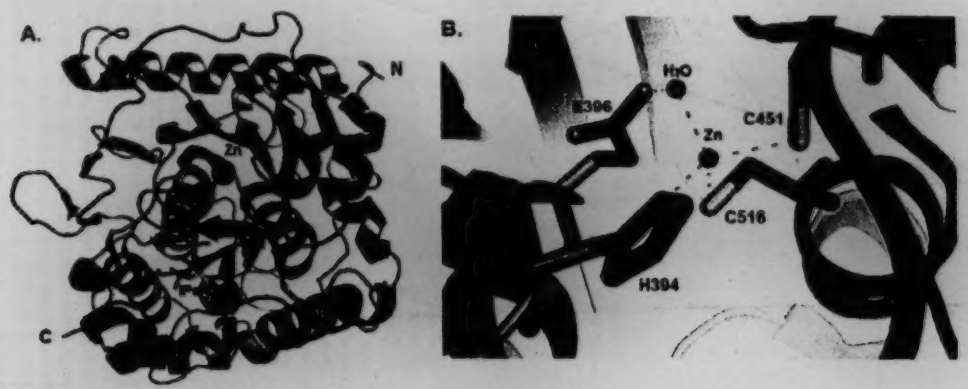

Figure 3-2. The X-ray structure of the catalytic domain of human ADAR2. (A) The ribbon model of the catalytic domain of human ADAR2. (B) Residue interactions at the active site. Zinc ion, coordinating residues (H394, E396, C451, C516) and the nucleophilic water are shown.

Among the three members of human ADAR family, ADAR1 and ADAR2 have been well characterized and ADAR3 is the most recent ADAR gene to be identified. Unlike ADAR1 and ADAR2, ADAR3 was shown to be able to bind known substrates of ADAR1 and ADAR2 but was reported to have no editing activity (17). As mentioned above, ADAR3 has a conserved C-terminal deaminase domain. The primary sequence alignment of the deaminase domains of ADAR1, ADAR2 and ADAR3 (Figure 3-3) shows that ADAR3 deaminase domain contains all the residues forming the catalytic center and coordinating the IP_6 molecule, which means that ADAR3 deaminase domain can be catalytically active.

To test the activity of ADAR3 deaminase domain *in vitro*, we cloned, over-expressed and purified the ADAR3 deaminase domain protein from human HEK

293 cells and conducted editing experiments. Meanwhile, to primarily understand the question that whether ADAR3 N-terminal domains affect its activity, we conducted an experiment to fuse ADAR2 N-terminal domains with ADAR3 deaminase domain and test the fusion protein's activity *in vitro*.

Figure 3-3. Sequence alignment of deaminase domains of human ADAR1, ADAR2 and ADAR3. Amino acid numbers are indicated at left. Red boxes: residues forming the catalytic center. Blue boxes: residues coordinating IP_6 molecule.

EXPERIMENTAL METHODS

Construction of fusion protein, ADAR2/3

The ADAR2/3 fusion was constructed by fusing residues 1-298 of ADAR2 with residues 342-739 of ADAR3. The fused ADAR2/3 DNA was cloned into mammalian

expression vector, p3XFLAG-CMV™-10 (Sigma), using Nhe I and BamH I sites. Primers used are listed as below:

ADAR2 1-298 F	5′-GCGCGCTCTAGAATGGATATAGAAGATGAAGAA-3′
ADAR2/ADAR3 1-298 R	5′-GCCGGGCATCTGGATGTTAAAAATGGCGGCCAG-3′
ADAR2/ADAR3 342-739 F	5′-CTGGCCGCCATTTTTAACATCCAGATGCCCGGC-3′
ADAR3 342-739 R	5′-GCGCGGATCCCTAGAGAGTCAGTAGA-3′

Cloning ADAR3 deaminase domain into p3XFLAG-CMV™-10 vector

The ADAR3 deaminase domain DNA was amplified from the ADAR3 gene and cloned into mammalian expression vector, p3XFLAG-CMV™-10 (Sigma), using Hind III and Xba I. Primers are listed below:

ADAR3 Deaminase Forward	5′-GCAAAAGCTTATCCAGATGCCCGGCCAC-3′
ADAR3 Deaminase Reverse	5′-GCGCGTTCTAGACTAGAGAGTCAGTAGAAACTGC-3′

Cell culture

Human HEK 293 cells were used for protein expression and purification. HEK 293 cells were cultured as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS at 37°C and 5% CO_2.

Protein expression and purification

ADAR2/3 and ADAR3 deaminase domain DNA were cloned into the p3XFLAG-CMV™-10 vector for over-expression. Cells were plated in 10 cm tissue

culture dishes and transiently transfected with 8 μg plasmid DNA using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol and harvested 24 hours post-transfection. Cells were incubated in 500 μl lysis buffer (50 mM Tris-HCl, 750 mM NaCl, 1 mM EDTA, 1% Triton-X100, pH 7.4) for 30 min and centrifuged for 10 min at 150,000 g. The supernatant was then incubated with 20 μl ANTI-FLAG® M2 affinity gel (Invitrogen) (treated following manufacturer's protocol) for 2 hours and centrifuged for 30 sec at 8,000 g and the supernatant was discarded. The tagged protein was eluted by incubating the gel with 40 μl wash buffer (50 mM Tris, 750 mM NaCl, pH 7.4) with 150 ng/μl 3 X FLAG peptide for 30 min and centrifuged for 1 min at 8,000 g.

To create stable cell lines expressing recombinant proteins, cells were plated and transfected as described above. 200 μl G418 disulfate salt solution (50 mg/ml) was added into the medium 24 hours post-transfection. The medium containing G418 was changed every 3-4 days for two weeks. Single colonies of cells were plated in 10 cm tissue culture dishes separately to examine the expression level of recombinant proteins.

RNA synthesis

Templates for the 78 nucleotide R/G pre-mRNA were generated by PCR from a substrate cloned into the pBS vector (Stratagene). [α-^{32}P] body-labeled 78 nucleotide R/G pre-mRNA was generated in a 25 μL transcription carried out in transcription buffer containing 40 mM Tris, pH 8.0, 6 mM $MgCl_2$, 100 mM NaCl, 10

mM DTT, 2 mM spermidine, 500 μM of each UTP, CTP and GTP, 15 μM of ATP, 1 U/mL of RNase Out (Invitrogen), 100 μCi α-^{32}ATP (Perkin Elmer), 0.1 μM DNA template and 2 U of T7 RNA polymerase (Invitrogen). Transcriptions were incubated at 37°C for 4 hours.

Editing assay

Editing reactions were carried out in 20 mM Hepes pH 8.0, 100 mM KCl, 0.5 mM DTT, 20% glycerol, 0.01% Nonidet P-40. RNA substrate (0.5 nM) was incubated with 10 pmol of each protein was used in the experiments. The substrate RNA was denatured at 80°C for 2 min and allowed to renature at room temperature for 30 min just prior to addition to the editing reactions. The reactions were quenched by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform followed by ethanol precipitation. The pellet was resuspended in 19 μl of double distilled water and digested with addition of 1 μl Nuclease P1 (1 μg/μl), and the products were resolved by thin layer chromatography (saturated $(NH_4)_2SO_4$: 0.1 M NaOAc: isopropanol; 79:19:2, v/v) using cellulose-polyethyleneimine chromatography plates. The TLC plates were exposed to a Molecular Dynamics Phosphor screen which was then scanned using a Molecular Dynamics Storm 860 PhosphorImager. The extent of editing was quantified using ImageQuant 5.0 software.

RESULTS

1. Over-expression of human ADAR3 deaminase domain and ADAR2/3

The deaminase domain of ADAR3 (ADAR3D) and fusion protein, ADAR2/3, were cloned into p3XFLAG-CMV™-10 vector, over-expressed and purified from human HEK 293 cells (Figure 3-4).

Figure 3-4. Over-expression of human ADAR3 deaminase domain and fusion protein ADAR2/3 in HEK 293 cells. The purified ADAR3 deaminase protein and ADAR2/3 fusion protein were run in 10% SDS-PAGE and stained with Brilliant Blue G-Colloidal (Sigma). The protein bands of correct size are indicated by red boxes. Lane 1: ADAR2 (76 KD). Lane 2: ADAR2/3 (76 KD). Lane 3: ADAR3 deaminase domain (44 KD). Molecular weight is indicated at left.

2. *In vitro* activity assay of ADAR3 deaminase domain and ADAR2/3

An *in vitro* activity assay of human ADAR3 deaminase domain and ADAR2/3 purified from human HEK 293 cells on 78-nucleotide R/G substrate (Figure 3-5) was performed (Figure 3-6). An activity assay of full-length ADAR2 purified from yeast (Sieminska, E., Schellenberg M., MacMillan, A.M., unpublished results) was performed at the same time as a control (Figure 3-6). The full-length ADAR2 exhibits

an editing efficiency of 4.8% on the substrate. ADAR3 deaminase domain and the fusion protein, ADAR2/3, exhibit little editing efficiency if any.

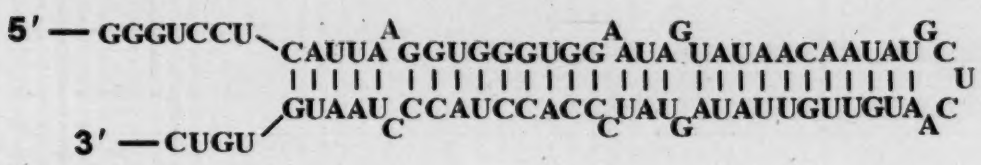

Figure 3-5. 78-nucleotide R/G substrate. The editing adenosine is labelled as red.

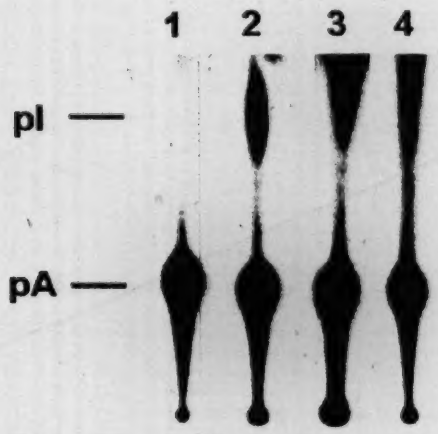

Figure 3-6. Editing assay of ADAR2/3 and ADAR3 deaminase protein. Lane 1: no protein control. Lane 2: full-length ADAR2. Lane 3: ADAR2/3 fusion protein. Lane 4: ADAR3 deaminase.

DISCUSSION

ADAR3 is a very special member of the human ADAR family. Although it has a similar domain structure to ADAR1 and ADAR2, it has been reported to be inactive on known substrates. However, the sequence alignment of the C-terminal catalytic domain, the deaminase domain, of ADAR1, ADAR2 and ADAR3 shows that ADAR3 deaminase domain contains all the residues that form the catalytic center and coordinate an IP_6 molecule. The SAXS (small angle X-ray scattering) models of the

deaminase domains of ADAR2 and ADAR3 (Sieminska, E., Schellenberg, M., MacMillan, A.M., unpublished data) show that the deaminase domains of ADAR2 and ADAR3 adapt similar tertiary structure (Figure 3-6), suggesting that ADAR3 deaminase domain may be catalytically active. The structural features that make ADAR3 act differently from ADAR1 and ADAR2 may not reside in the deaminase domain. Compared to ADAR2, ADAR3 has an arginine-rich domain at the N-terminus, which was suggested to be responsible for binding to single-stranded RNA (17). Our *in vitro* editing assay shows that ADAR3 deaminase domain did have little activity on a known ADAR substrate (R/G substrate) compared to that of full-length ADAR2, which suggests that the N-terminal domains are important for editing activity of ADARs. The fusion protein, ADAR2/3, exhibited a better editing activity than that of ADAR3 deaminase domain, but again lower than that of full-length ADAR2. Thus, although ADAR3 deaminase domain has all the conserved residues of the catalytic center and the binding to IP_6 molecule, somehow it is different from ADAR2 deaminase domain because the replacement of ADAR3 N-terminus with ADAR2 N-terminus did not make the fusion protein, ADAR2/3, act the same as full-length ADAR2. It is still unclear which N-terminal domains affect ADAR3's activity and specificity that make the protein different from ADAR1 and ADAR2. To get the crystal or SAXS structure of full-length ADAR3 will help to answer such question. Although the SAXS data of ADAR2 deaminase domain and ADAR3 deaminase domain showed that they adopted similar tertiary structure, the editing assay results of ADAR2 and ADAR2/3 indicate the two deaminase domains

do not act exactly the same, suggesting that there are some other important residues outside the conserved catalytic center and IP_6 binding motif affecting the activity and specificity of ADAR.

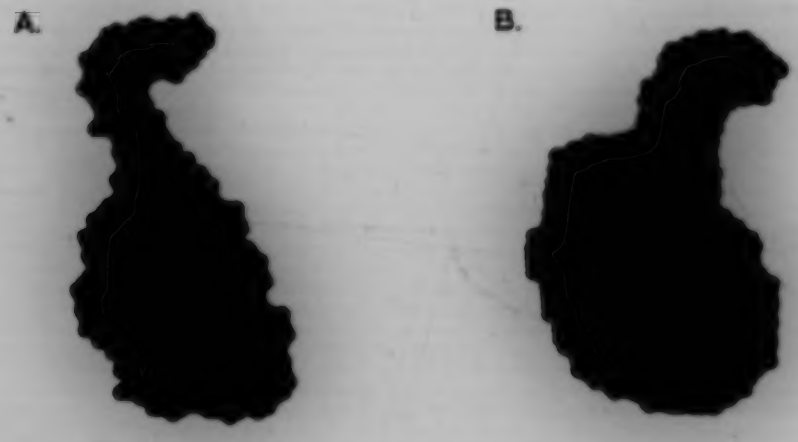

Figure 3-7. SAXS model of deaminase domains of ADAR2 and ADAR3. A. ADAR2 deaminase domain. B. ADAR3 deaminase domain.

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